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SSC1, an Essential Member of the Yeast HSP70 Multigene Family, Encodes a Mitochondrial Protein

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***SSC1* is an essential member of the yeast HSP70 multigene family (E. Craig, J. Kramer, and J. Kosic-Smithers, Proc. Natl. Acad. Sci. USA 84:4156–4160, 1987). Analysis of the *SSC1* DNA sequence revealed that it could encode a 70,627-dalton protein that is more similar to DnaK, an *Escherichia coli* hsp70 protein, than other yeast hsp70s whose sequences have been determined. Ssc1p was found to have an amino-terminal extension of 28 amino acids, in comparison with either Ssa1p, another hsp70 yeast protein, or Dnak. This putative leader is rich in basic and hydroxyl amino acids, characteristic of many mitochondrial leader sequences. Ssc1p that was synthesized in vitro could be imported into mitochondria and was cleaved in the process. The imported protein comigrated with an abundant mitochondrial protein that reacted with hsp70-specific antibodies. We conclude that Ssc1p is a mitochondrial protein and that hsp70 proteins perform functions in many compartments of the cell.**

The 70-kilodalton (kDa) heat shock proteins (hsp70s) are among the most highly conserved proteins known. Related proteins have been found in all species analyzed, from bacteria to yeast, insects, arctic fishes, and humans (for a review, see reference 18). The bacterium *Escherichia coli* has a single hsp70-related protein, the product of the heat-inducible *dnaK* gene (1). Most if not all eucaryotic cells, including those of humans, fruit flies, and yeasts, have a family of HSP70-related genes (5, 16, 29). Some members of these multigene families are inducible by stresses such as a heat shock or treatment with chemicals; some are present during normal cellular growth. Studies in *Drosophila melanogaster* and human cells have shown that the major heat-inducible member of the family is translocated into the nucleus upon a heat shock and, after the stress has passed, moves back into the cytoplasm (28). Another member of the mammalian family, grp78 or immunoglobulin heavy-chain binding protein (BiP), is found in the endoplasmic reticulum (ER) (19). All of the hsp70 proteins identified thus far have a high affinity for ATP (2, 18, 31). It has been proposed that hsp70-related proteins are involved in disrupting or altering protein-protein interactions in an ATP-dependent fashion (22).

The yeast HSP70 multigene family is composed of at least nine members. The sequence relationships among the members of the family are complex, with nucleotide similarities ranging from about 50 to 97%. Eight of these genes isolated in our laboratory have been designated stress seventy genes and divided into four subgroups, A through D, on the basis of structural and functional criteria; the genes are *SSA1* through *-4*, *SSB1* and *-2*, *SSC1*, and *SSD1* (4, 6, 33). Another member of the family, *KAR2*, has recently been identified (M. Rose, L. Misra, and J. Vogel, Cell, in press).

The yeast HSP70 multigene family encodes proteins with essential functions. The *SSA* subfamily is an essential subfamily. Recent evidence indicates that products of these genes play a role in the translocation of proteins from the cytoplasm into both the ER and mitochondria (3, 8). *KAR2* is an essential gene. Kar2p is related to grp78 of mammalian cells, which is found in the ER (Rose et al., in press).

SSC1, also an essential gene (4), is expressed in moderate amounts under normal growth conditions. *SSC1* RNA levels increase about 10-fold within 10 min of a shift from 23 to 37°C (32). We determined the DNA sequence of *SSC1* and found that the predicted amino terminus of the protein had characteristics of a mitochondrial leader sequence. Additional experiments were carried out to show that Ssc1p is a mitochondrial protein.

MATERIALS AND METHODS

Strains and culture conditions. The wild-type *Saccharomyces cerevisiae* strain D273-10B (ATCC 25657), used for preparation of mitochondria for in vitro import assays, was grown at 30°C in semisynthetic medium containing 3% glycerol and 2% lactate (7). DS10 (a *leu2-3,112 lys1 lys2 his3-11,15 Δtrp1 ura3-52*) was used for preparation of mitochondrial and total-protein samples.

Plasmid construction. To construct an *SSC1* clone that could be used for in vitro synthesis of *SSC1* RNA, clone pSSC1G, containing the entire *SSC1* gene on a 6.1-kilobase *Bg*lII fragment in the *Bg*lII site of pMT11 (4), was used as the starting material for digestion with exonuclease III. *Bam*HI linkers were added to the digested DNA, and the DNA was religated. A clone containing a *Bam*HI site at position −70 (with +1 being the A of the initiating ATG) was then cloned on a *Bam*HI-*Eco*RI fragment into the expression vector pGEM32II(+) (Promega Biotech), placing *SSC1* near the SP6 promoter and generating plasmid pSP6-SSC1.

DNA sequencing and sequence comparison. The DNA sequence of *SSC1* was determined by the dideoxy-chain termination method (24), using the Sequenase enzyme system (U.S. Biochemical Corp.) and [^{35}S]dATP (Dupont, NEN Research Products) according to the instructions of the manufacturers. Sequencing clones were generated by cloning fragments generated by exonuclease III digestion, as described above, into M13. In some cases, after the se-

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quence of one strand was determined, oligonucleotides homologous to *SSC1* DNA were synthesized and used as primers. The entire sequence was determined on both strands, and all sequenced restriction sites were crossed.

The amino acid sequences were aligned by using the program GAP (9). GAP uses the algorithm of Needleman and Wunsch (20), modified to allow the imposition of a gap-length penalty. Conservative replacements are based on the evolutionary distance between amino acids as measured by Schwartz and Dayhoff (25) and normalized by Gribskov and Burgess (12). By this method, identities are given a value of 1.5. Conservative replacements are defined as those having a value of ≥0.5. In this study, percentage identity is defined as the number of identical amino acids observed after alignment of the two sequences is divided by the number of residues in the shorter sequence.

Preparation of mitochondria. Mitochondria were prepared essentially as described by Daum et al. (7). Briefly, cells were harvested and washed in SH (1.2 M sorbitol, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.4]) and suspended in the same solution plus 0.8% 2-mercaptoethanol. Cells were then digested with zymolyase-100T (1 mg/g [wet weight] of cells; ICN Immunobiologicals) at room temperature with gentle shaking. Conversion to spheroplasts was checked by testing for lysis in 1% sodium dodecyl sulfate. The spheroplasts were harvested and washed two times in SH. For homogenization, cells were suspended in MH (0.6 M mannitol, 10 mM HEPES [pH 7.4]) plus 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mg of alpha-2-macroglobulin per ml. All operations from this point on were carried out on ice. Spheroplasts were homogenized by 10 strokes in a tight-fitting Dounce homogenizer. The homogenate was spun for 5 min at 3,500 rpm in an SS34 rotor (Ivan Sorvall, Inc.). The pellet was suspended and homogenized again. After centrifugation, the supernatants were pooled, and cell debris was pelleted by spinning for 5 min at 3,500 rpm. The supernatant was then centrifuged at 9,000 rpm for 10 min to recover the mitochondria. After resuspension, the sequential pelleting of cell debris and mitochondria was repeated three times, and the mitochondria were suspended at 10 mg/ml in SEM (250 mM sucrose, 10 mM morpholinepropanesulfonic acid [MOPS; pH 7.2], 1 mM EDTA) or MH.

In vitro synthesis of Ssc1p and import into mitochondria. pSP6-SSC1 was cleaved with *Eco*R1 to form a linear template. RNA was synthesized by using SP6 polymerase (Promega Biotech) according to the instructions provided. The RNA was added to a rabbit reticulocyte in vitro translation system (Promega Biotech) in the presence of [^{35}S]methionine (1,200 Ci/mmol; translation grade; Dupont, NEN). Import into mitochondria was carried out essentially by the method of Gasser et al. (11). Import reactions contained 15 μl of reticulocyte lysate containing [^{35}S]Ssc1p (approximately 600,000 cpm), 25 μl of mitochondria at 10 mg/ml (as measured at A_{260}), 100 mM KCl, and an energy mix as previously described (11). When used, valinomycin was added to the energized mitochondria 2 min before the addition of lysate to give a final concentration of 1.0 μM. The mixture was incubated for 1 h at 30°C, and then the mitochondria were pelleted through a 1-ml sucrose cushion (20% sucrose, 100 mM KCl, 2 mM $MgCl_2$, 20 mM HEPES [pH 7.4]). If protease treatment was performed, the resuspended mitochondria were incubated with agarose-bound trypsin (Sigma Chemical Co.) for 45 min at 30°C at pH 8.0 or with 250 μg of proteinase K per ml for 30 min at 30°C. The trypsin beads were removed by centrifugation, and 1 mM PMSF and 1 mM tosyl-L-lysyl chloromethyl ketone (TLCK) were added. One-dimensional and two-dimensional polyacrylamide gel electrophoresis was performed as previously described (33).

Antibody production and Western blot (immunoblot) analysis. An 11-amino-acid peptide of the sequence NH_2-Val-Gly-Ile-Asp-Leu-Gly-Thr-Thr-Tyr-Ser-Cys-COOH was synthesized at the University of Wisconsin Biotechnology Center and injected directly into rabbits to generate antibodies. This sequence is identical to the amino acids of the *SSA1* protein from positions 5 through 15. Antibodies to hexokinase and citrate synthetase were a generous gift of G. Schatz.

Electrophoretic transfer of proteins to membrane filters was carried out as described by Towbin et al. (27). Transfer was onto an Immobilon membrane (Millipore Corp.) was carried out for 1.5 h at a current of 1 A. After transfer, filters were blocked for 30 min in 50 mM Tris (pH 7.5)–180 mM NaCl (TBS) plus 0.05% Tween and for 60 min in 50 mM Tris (pH 9.0)–180 mM NaCl–0.05% Tween–1% bovine serum albumin. Before incubation with primary antibody, filters were washed in TBS-Tween buffer. Primary-antibody incubation with the anti-hsp70 conserved-region peptide was carried out for 2 h in TBS–1% bovine serum albumin, using a 1:50 dilution of serum. After incubation, filters were washed twice in TBS-Tween buffer. Primary-antibody reaction was visualized by either protein A binding or incubation with a secondary antibody conjugated to alkaline phosphatase (Sigma). All incubations were carried out at room temperature.

To visualize the antibody-antigen reaction using protein A, filters were incubated in a 1:500 dilution (in TBS–1% bovine serum albumin) of ^{125}I-labeled, affinity-purified protein A (Amersham Corp.) for 1 h. Filters were then washed two times for 40 min each in TBS-Tween and briefly rinsed again in TBS. Filters were air dried before autoradiography. Alternatively, primary-antibody reaction was visualized by incubating filters with a 1:1,000 dilution of goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase in TBS–1% bovine serum albumin, using the protocol of Jane Cooper (personal communication). After 1 h of incubation, the filters were removed and washed twice in TBS-Tween with vigorous agitation. To develop the color, filters were incubated in a solution of TBS containing 0.33 mg of nitroblue tetrazolium (Sigma) per ml and 0.165 mg of 5-bromo-4-chloro-3-indolylphosphate (toluidine salt; Sigma) until the filter itself started to turn light purple. At this time, filters were removed and washed with distilled water. The development solution was prepared fresh just before use by addition of concentrated stock solutions of the substrates (50 mg of nitroblue tetrazolium per ml in 70% dimethyl formamide and 50 mg of 5-bromo-4-chloro-indolylphosphate per ml in 100% dimethyl formamide) to TBS. Color development typically reached maximal levels in less than 5 min.

ATP-agarose chromatography. Isolation of ATP-binding proteins was carried out essentially as described by Welch and Feramisco (31). To prepare yeast whole-cell lysates, 5×10^8 cells were combined with 0.5 ml of glass beads (type V; Sigma) and 1 ml of buffer A (50 mM HEPES-KOH [pH 7.5], 25 mM KCl, 2 mM magnesium acetate, 15 mM β-mercaptoethanol, plus the protease inhibitors PMSF [1 mM], TLCK [100 μg/ml], and pepstatin A [1.4 μg/ml]) in a 1.5-ml microfuge tube. Disruption was carried out on a Biospec Products Mini-bead Beater for 3 min, with cooling on ice after each minute. After disruption, the tube was centrifuged briefly to pellet glass beads and undisrupted cells. The

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ATCGCAATGG TACAATGTGC TAAAGCTAGT GCCTTAGATG CGCTGAATAA TTGTATATCA CCCACAACGG CAGAGCTTTT CCATGGGCTT CGGCCCGGTT CCACCGCCCC CTGCCTCTTT   -241
TTCCGGTGAC CGCTTACGGT GCGGGTATA AAAACGAACG AICCAACGCC GGCTCGACGC TTGGGAACTT TTGGGTTCTT TGTTCCACGT TTGAAAACTC CAATACGTTG CTGTCCTCCT   -121
CTGCCTGCTG TACATATCGA CGACCGCTGC TCACCAACCT TTGCCAGAGT GACAACTITT GTATTTACGA CCCATTTAAT ATAATTAAGA CTACACAAAT CTCATATTAT ACGCACCAAG    -1
ATGCTTGCTG CTAAAAACAT ACTAAACAGG TCAAGCTTGT CTAGCTCTTT CCGTATTGCC ACACGTTTGC AGTCAACCAA GGTTCAAGGT TCCGTCATCG GTATCGATTT GGGTACCACC    120
AACTCTGCGG TTGCCATTAT GGAAGGTAAA GTTCCAAAAA TTATTGAAAA CGCCGAAGGT TCCAGAACTA CTCCTTCTGT AGTAGCTTTC ACTAAAGAGG GAGAACGTTT GGTTGGTATT    240
CCAGCCAAGC GTCAAGCCGT AGTCAACCCA GAAAACACCC TATTTGCTAC CAAGCGTTTG ATTGGTCGTC GTTTCGAAGA CGCTGAAGTG CAAAGAGATA TCAAGCAAGT TCCATACAAG    360
ATCGTCAAGC ACTCCAACGG GGATGCTTGG GTTGAGGCCA GAGGTCAAAC TTACTCACCA GCCCAAATCG GTGGGTTCGT CTTGAACAAG ATGAAGGAAA CAGCTGAGGC CTACTTGGGT    480
AAGCCAGTTA AGAATGCTGT TGTCACTGTC CCAGCTTATT TCAACGACTC TCAAAGACAA GCTACTAAAG ACGCAGGCCA AATTGTTGGT TTGAACGTTT TACGTGTCGT CAATGAACCA    600
ACCGCCGCTG CCTTAGCTTA CGGTTTGGAA AAATCCGACT CTAAAGTTGT TGCCGTTTTC GATTTGGGTG GTGGTACTTT CGATATCTCC ATCTTAGATA TTGACAACGG TGTTTTTGAA    720
GTTAAGTCCA CTAACGGTGA CACTCATTTG GGTGGTGAAG ATTTCGACAT CTATTTGTTG AGAGAGATTG TTTCTCGTTT CAAGACGGAA ACTGGTAATG ATTTGGAAAA TGACCGTATG    840
GCTATCCAAA GAATTAGAGA AGCTGCTGAA AAGGCTAAGA TTGAGCTATC TTCTACCGTT TCCACTGAAA TCAACCTGCC ATTTATCACT GCTGATGCCT CAGGTCCAAA GCATATCAAC    960
ATGAAGTTCT CCAGGGCTCA ATTCGAGACT TTGACAGCCC CACTAGTTAA GAGAACTGTC GACCCAGTCA AGAAGGCTTT GAAAGACGCC GGTTTGTCTA CTTCAGACAT ATCTGAAGTC   1080
TTATTGGTCG GTGGTATGTC CAGAATGCCT AAGGTTGTCG AAACCGTTAA ATCTTTGTTT GGTAAGGACC CATCTAAGGC CGTCAACCCA GATGAAGCTG TTGCCATTGG TGCTGCTGTG   1200
CAAGGTGCTG TCTTGTCCGG TGAGGTTACT GACGTCTTAT TATTAGATGT TACCCCATTG TCTCTAGGTA TCGAAACTTT AGGTGGTGTT TTCACAAGAT TGATTCCAAG AAACACTACT   1320
ATTCCAACAA AGAAATCTCA AATCTTCTCC ACTGCCGCTG CTGGTCAAAC TTCTGTTGAA ATCAGAGTTT TCCAAGGTGA AAGAGAATTG GTTAGAGACA ACAAATTGAT TGGTAACTTC   1440
ACTTTAGCCG GTATCCCACC TGCTCCAAAG GGTGTCCCAC AAATCGAAGT CACTTTTGAC ATCGATGCCG ATGGTATTAT TAACGTTTCT GCTAGAGACA AGCTACAAA CAAAGATTCT   1560
TCTATTACTG TTGCCGGTTC TTCTGGTTTG TCGAAAACG AAATTGAACA AATGGTTAAC GACGCTGAAA AATTCAAGTC TCAAGATGAA GCTAGAAAAC AAGCCATGGA AACTGCCAAC   1680
AAGGCTGACC AATTGGCCAA CGATACTGAA AACTCCTTGA AAGAATTGA AGGTAAGGTT GACAAGGCTG AAGCCCAAAA GGTTAGGGAT CAAATCACTT CCTTGAAGGA GTTGGTTGCT   1800
AGAGTACAAG GTGGCGAAGA GGTTAACGCT GAGGAGTTAA AGACCAAGAC CGAAGAATTG CAAACTTCCT CGATGAAATT GTTTGAACAA TTATACAAGA ACGACTCTAA CAACAACAAC   1920
AACAACAACG GCAACAATGC CGAATCTGGT CGAAACTAAGC AGTAAAAAGC AAATTCCTGT TAATAAATTA CTACCACAAT GTTAAACTAG AAAGAATGAC AAAAAACATA ATAATAACTA   2040
ACCAGTATTT TTGTTTCTTT ATTTTTAAG AACATTTATA TACATAATAT ACACGCCCCA AACATATATC GTTTATATAA GTACTGTTTC CTCGCATTAA GCGGGAAAGC CATGTATCTC   2160
TTGTAATTAG TTTGATCCTG TTCTATACCC AAATGTACTC CTCAAAATTA TTACATTTTA TTATGCCTTT TTCTTAGATG GGCTTTCACT CCACTTTACG AAAAACTGAG AAAAAAAGAG   2280
TAGGAGAAAA ACTAGAAAAG GGGAATCACA CACACACACA CACACACACA CACACACGTC TAATATCGGC GCATATTTAT CAAATCTAAA AGGCAAAGAA TCTTAATACA   2400
TAAAATCCCA GACGGTAATG ATG                                                                                                   2423
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FIG. 1. Nucleotide sequence of the *SSC1* gene. All of the sequence was determined on both strands of the DNA by the dideoxy-chain termination method (24). The initiation and termination codons of the open reading frame encoding Ssc1p are double underlined. The A of the initiating ATG is designated as position 1. In the 5′ noncoding region, sequences containing similarity to the heat shock element consensus sequence CNNGAANNTTCNNG (21) are underlined, with asterisks denoting mismatches.

supernatant was withdrawn and centrifuged for 5 min to further pellet debris. Whole-cell lysate from the equivalent of 3×10^9 cells (typically 6 mg of protein) was loaded onto a 1.5-ml ATP-agarose column (A-2767; Sigma) at a flow rate of approximately 1 ml/3 to 4 min. The column was then sequentially washed with 3.5 void volumes of buffer A, 3.5 void volumes of buffer A–1 M KCl, 3.5 void volumes of buffer A, and 3.5 void volumes of buffer A–2 mM GTP. To elute fractions, 4 void volumes of buffer A plus the appropriate concentration of ATP was used. ATP-eluted fractions were concentrated by acetone precipitation and then suspended in two-dimensional gel lysis buffer for application onto tube gels. For the comigration experiment, imported Ssc1p was mixed directly with the resuspended ATP-eluted fractions before isoelectric focusing.

RESULTS

DNA sequence of *SSC1*. The *SSC1* gene had previously been isolated and the mRNA coding region had been mapped by a combination of complementation and hybridization analyses (4). The DNA sequence of the mRNA homologous region was determined (Fig. 1) and found to contain an open reading frame capable of encoding a 70,627-Da protein (Fig. 2) highly similar to the proteins of the hsp70 family. In the 360 nucleotides 5′ of the open reading frame, there were three regions containing two or three mismatches (Fig. 1) to the heat shock element previously shown to be responsible for heat-inducible transcription of a number of genes (22). One or more of these sequences may be responsible for the heat-inducible increase of *SSC1* RNA levels.

The predicted protein encoded by *SSC1* is more closely related to the *E. coli dnaK* protein than to any other hsp70 protein whose sequence has been determined (Fig. 2; Table 1). Ssc1p is 58% identical to DnaK, 50% identical to Ssa1p, 48% identical to Ssb1p, and 48% identical to rat grp78. One region, between amino acids 272 and 313 of Ssc1p, shows a much higher degree of similarity to DnaK than to Ssa1p. It is 88% related to DnaK (71% identity) but only 52% related to Ssa1p (31% identity). No other stretch of amino acids shows a greater disparity in values, suggesting that this region may be involved in an organelle or procaryotic specific function.

Unlike other hsp70 proteins, the predicted *SSC1* protein contains a string of asparagine residues near the carboxy terminus. Of the 10 amino acids from positions 637 to 646, 9 are asparagine. These residues make the carboxy terminus of Ssc1p much more hydrophilic than the carboxyl termini of other hsp70 proteins.

Most interestingly, Ssc1p contains an additional 28 amino acids at the amino terminus as compared with Ssa1p and DnaK. This putative leader sequence is positively charged, containing three arginine and two lysine residues, and no acidic amino acids. The basic amino acids are spaced three to seven amino acids apart. The leader contains six serine and two threonine residues. Many amino-terminal presequences of proteins that are translocated into mitochondria are rich in basic and hydroxyl amino acids. The basic amino acids are usually spaced three to five amino acids apart (10).

FIG. 2. Comparison of the predicted amino acid sequence of the *SSC1* protein with those of the *dnaK* and *SSA1* proteins. Symbols: ●, amino acids in the *SSC1* protein that are identical to those in the *dnaK* or *SSA1* protein; ○, replacements by conserved amino acids; . . . , gaps inserted to improve the alignment. The *dnaK* and *SSA1* sequences were taken from Bardwell and Craig (1) and Slater and Craig (25a), respectively. In the leader sequence, hydroxylated amino acids are underlined, and basic amino acids are indicated by a plus sign.

From this analysis, we suspected that Ssc1p was a mitochondrial protein.

Import of Ssc1p into mitochondria in vitro. Since the characteristics of the putative leader sequence suggested that Ssc1p may be a mitochondrial protein, we decided to test whether Ssc1p could be imported into mitochondria in vitro. *SSC1* mRNA was synthesized in vitro and added to a reticulocyte translation system in the presence of [^{35}S]methionine. The labeled protein was incubated with energized, isolated yeast mitochondria. Ssc1p was imported into mitochondria, as demonstrated by its cosedimentation with mitochondria and resistance to externally added protease in the absence of detergent (Fig. 3A). However, upon disruption of the mitochondria by detergent, Ssc1p was degraded by protease.

When mitochondria were deenergized by the addition of valinomycin plus potassium, import *SSC1* precursor and its cleavage to a smaller product were prevented (Fig. 3B). Only a small amount of precursor was associated with the mitochondria, and this was susceptible to trypsin digestion. This inhibition of import is consistent with translocation of Ssc1p into mitochondria, since import into mitochondria requires an electrochemical gradient across the inner membrane (11).

The relative mobility of Ssc1p in sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis changed after import, providing evidence that Ssc1p is synthesized as a precursor molecule. The difference between the imported protein and the precursor form was more obvious when the proteins were analyzed on two-dimensional gels (Fig. 4) because only a few thousand daltons were removed from the relatively large precursor protein (of about 70,000 Da) and because of the presence of incomplete translation products in the lysate. The mature, imported form, as expected, was smaller and more acidic than the precursor. This shift in charge is predicted on the basis of the structure of the putative precursor, which contains several basic but no acidic residues.

Ssc1p is an abundant protein in mitochondria. The results presented above show that Ssc1p can be imported into mitochondria. To directly demonstrate that Ssc1p is a mitochondrial protein, mitochondria were isolated from DS10 cells, and mitochondrial proteins were analyzed. DS10 was

TABLE 1. Relationships between Ssc1p and other hsp70 proteins

Protein	Relationship to:							
	DnaK		Ssa1p		grp78		Ssb1p	
	% Amino acid identity	% Amino acid similarity[a]	% Amino acid identity	% Amino acid similarity	% Amino acid identity	% Amino acid similarity	% Amino acid identity	% Amino acid similarity
Ssc1p	57.8	73.0	50.3	67.3	47.7	65.8	48.2	65.2
DnaK			49.8	67.1	49.1	66.4	46.4	64.2
Ssa1p					64.0	77.7	60.4	74.7

[a] See Materials and Methods.

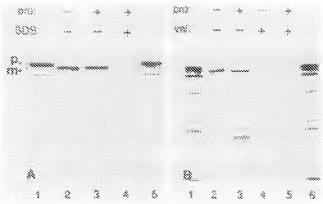

FIG. 3. Import of Ssc1p into mitochondria. The *SSC1* protein was synthesized in a reticulocyte lysate in the presence of [^{35}S]methionine (lanes 1 and 5 [A] and 1 and 6 [B]; these lanes contained 67% of the radiolabeled protein that was incubated with mitochondria) and incubated for 30 min at 30°C with isolated mitochondria (lanes 2). (A) Incubation in the presence of proteinase K (lane 3) and proteinase K plus SDS (lane 4); (B) incubation in the presence of trypsin (lane 3), valinomycin and potassium (lane 4), and trypsin, valinomycin, and potassium (lane 5). The samples were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. p, Precursor; m, mature.

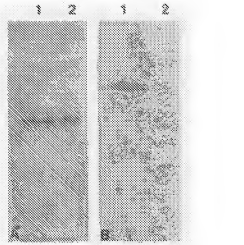

FIG. 5. Assessment of mitochondrial purity. Proteins prepared from whole cells (lanes 1) and from mitochondria isolated from an equivalent number of cells (lanes 2) were electrophoresed in SDS-acrylamide gels. The proteins were transferred to filters and reacted with antibodies to citrate synthetase, a mitochondrial matrix protein (A), and hexokinase, a cytoplasmic protein (B). The filters were then incubated with [^{125}I]protein A and subjected to autoradiography.

used as a source of mitochondria because this strain is closely related to S288C, from which the *SSC1* clone was isolated. The purity of the isolated mitochondria was checked by using an antibody generated against hexokinase, a cytoplasmic enzyme. Protein samples prepared from whole cells and from mitochondria that had been isolated from an equivalent number of cells were subjected to Western blot analysis. Little cytoplasmic contamination was observed in the mitochondrial preparation (Fig. 5B), whereas the two preparations contained similar amounts of two mitochondrial proteins, citrate synthetase (Fig. 5A) and the β subunit of the F_1 ATPase (data not shown). To determine that Ssc1p was a mitochondrial protein, the isolated mitochondrial proteins were mixed with ^{35}S-labeled Ssc1p that had been imported into mitochondria in vitro and separated by two-dimensional polyacrylamide gel electrophoresis. Cleaved Ssc1p comigrated with a major mitochondrial protein of about 70,000 Da (Fig. 6). Both Ssc1p imported in vitro and that isolated from cells migrated as a series of isoforms identical in molecular weight but different in charge. This difference in charge suggests that Ssc1p is modified in some way.

Western blot analysis was carried out on proteins isolated from mitochondria and separated by two-dimensional gel electrophoresis, using antibodies generated to a peptide present in the amino-terminal region of many hsp70 proteins (Fig. 6C). The protein that comigrated with cleaved Ssc1p was also recognized by the hsp70 antibody, providing further evidence for the presence of Ssc1p in mitochondria. Another, much less abundant protein very similar in size to but slightly more acidic than Ssc1p also reacted with the antibody. It is likely that the antibody was reacting with a small amount of Ssa1p or -2p in the mitochondrial preparation, since this reactive protein migrated at a position expected for Ssa1p and -2p (33). This autoradiogram may have led to an

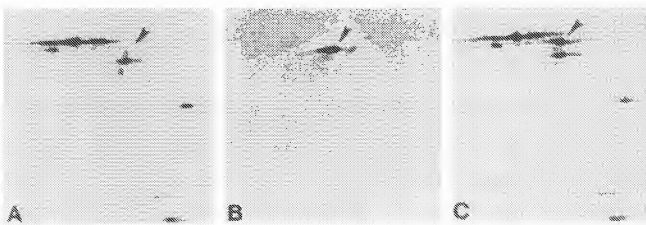

FIG. 4. Separation of the *SSC1* protein translated in reticulocyte lysates and imported into mitochondria in two-dimensional gels. (A) Translated protein; (B) protein imported into mitochondria; (C) mixture of the proteins run in panels A and B. The more acidic side of the gel is to the right; arrows indicate positions of imported protein.

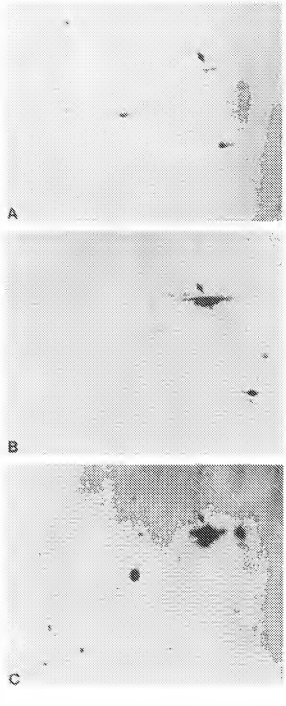

FIG. 6. Comigration of Ssc1p imported into mitochondria in vitro with a major mitochondrial protein. Unlabeled mitochondrial proteins isolated from DS10 were mixed with the *SSC1* protein, synthesized in vitro, imported into mitochondria, and separated in a two-dimensional gel. The gel was stained with Coomassie blue (A), dried, and subjected to autoradiography (B). A sample of DS10 mitochondrial proteins was electrophoresed in an identical manner, and the proteins were blotted to a filter. The proteins were then reacted with antibody directed against an oligopeptide present in the amino-terminal region of many hsp70 proteins (C) (see Materials and Methods). After incubation with antibody, blots were washed and incubated with [^{125}I]protein A. Arrows indicate positions of migration of the *SSC1* protein.

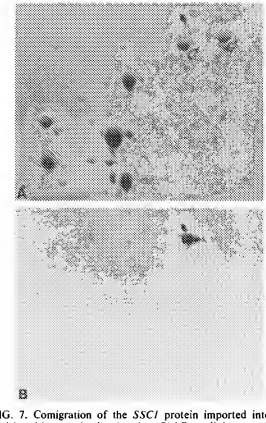

FIG. 7. Comigration of the *SSC1* protein imported into mitochondria with a modestly abundant 70-kDa cellular protein. The *SSC1* protein was translated in a reticulocyte lysate in the presence of [^{35}S]methionine and imported into mitochondria. Imported Ssc1p was mixed with cellular protein from DS10. The mixture of proteins was separated on a two-dimensional gel. The gel was stained with Coomassie blue (A), dried, and subjected to autoradiography (B). The more acidic side of the gel is oriented to the right. The solid arrow indicates the position of migration of in vitro imported *SSC1* protein; open arrows indicate positions of Ssa1p and -2p (right) and Ssb1p and -2p (left).

overestimation of the amount of the *SSA* proteins in the mitochondrial preparation, since the *SSA* proteins had complete identity with the immunogen, whereas Ssc1p did not (Fig. 2). Ssc1p has 3 mismatches of 11 with the immunogen and probably did not react with the antibody as strongly as did *SSA* proteins. However, seven contiguous amino acids in the *SSC1* protein, enough to constitute an epitope (26), are identical to those in the immunogen. An additional protein reacted weakly with the hsp70 antibody. Since this protein was the most abundant protein present in the gel, the reaction may have resulted from nonspecific interactions.

Identification of Ssc1p in total-cell extracts. To identify Ssc1p in whole-cell extracts, [^{35}S]Ssc1p that had been imported into mitochondria in vitro was mixed with total-cell protein from DS10. Mature Ssc1p comigrated with a moderately abundant protein of approximately 70 kDa (Fig. 7). Through analysis of previously constructed strains containing mutations in hsp70-related genes, we have been able to identify a number of stress seventy family members (33). Ssa1p, Ssa2p, and the combined spots of Ssb1p and -2p are indicated in Fig. 7. As expected from the predicted molecular weights of the proteins as determined from DNA sequence analysis, Ssc1p was slightly larger than Ssb1p, which has a predicted molecular weight of 66,601 Da. Ssc1p, which

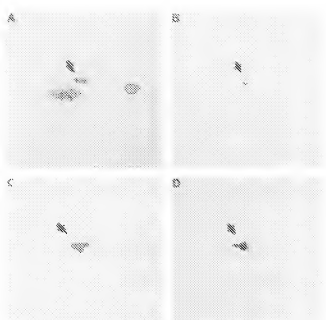

FIG. 8. Comigration of Ssc1p with an hsp70 ATP-binding protein. Total yeast crude lysates (A) or proteins isolated from purified mitochondria (C) were chromatographed on ATP-agarose and eluted with ATP. The eluted proteins were mixed with *SSC1* protein that had been translated in vitro in the presence of [^{35}S]methionine and imported into mitochondria. The mixture was separated by two-dimensional gel electrophoresis. The proteins were blotted onto nitrocellulose and reacted with hsp70-specific antibody (see Materials and Methods). The primary antibody was visualized by using alkaline phosphatase-conjugated goat anti-rabbit antibody, and the filter was photographed (A and C). Arrows indicate positions of migration of the *SSC1* protein isoforms. (B and D) Autoradiograms derived from exposure of the filter, indicating comigration of Ssc1p with an ATP-binding protein.

had a pI very close to that of Ssb1p, was slightly more basic than Ssa1p and -2p. Judging from the intensity of the stained spots, Ssc1p appeared to be present at about 10% the abundance of *SSA1* and -2 or *SSB1* and -2 proteins. In the protein preparations obtained from purified mitochondria, Ssa1p and -2p and Ssb1p and -2p were much less abundant than Ssc1p (Fig. 6). The relative increase of Ssc1p in purified mitochondria suggested that Ssc1p is predominantly localized in mitochondria.

Comigration of Ssc1p with an hsp70 ATP-binding protein. Most hsp70 proteins bind ATP and can be separated from most other proteins by chromatography on ATP-affinity columns. To determine whether Ssc1p could bind ATP, crude yeast cell extracts were loaded directly onto ATP-agarose columns, and the proteins were eluted with ATP. The eluted proteins were analyzed by two-dimensional gel electrophoresis and Western blotting (Fig. 8A). As expected, a major cluster of ATP-binding proteins with molecular sizes of 66 to 75 kDa migrated in the pI 5.1 to 5.7 range. These proteins reacted with the polyclonal antisera directed against the amino-terminal conserved region of hsp70 proteins. Labeled Ssc1p that had been imported into mitochondria comigrated with an antibody-reactive protein (Fig. 8B), suggesting that Ssc1p is an ATP-binding protein. Proteins isolated from purified mitochondria were also subjected to ATP-agarose chromatography and analyzed as described above. Mitochondria predominantly contained a single cluster of isoforms migrating at about 70kDa which reacted with hsp70-specific antibody (Fig. 8C). These isoforms comigrated with Ssc1p that had been imported into mitochondria, indicating that Ssc1p is an ATP-binding protein.

DISCUSSION

The results presented above indicate that Ssc1p is a mitochondrial protein. The predicted sequence of the amino-terminal region of the mature *SSC1* protein is similar to the sequences of a number of proteins imported into mitochondria in that it contains several basic and hydroxyl amino acids. Furthermore, in vitro-synthesized Ssc1p is cleaved upon import into isolated mitochondria, and this import is dependent on an electrochemical gradient. The imported protein comigrates with a major mitochondrial protein. In two-dimensional protein gels, the imported Ssc1p migrates as a series of isoforms with the same molecular weights but slightly different charges. The imported proteins comigrate with a series of isoforms present in mitochondria isolated in vivo. Ssc1p, like other hsp70 proteins, appears to be an ATP-binding protein, since a major mitochondrial 70-kDa protein that binds to ATP and reacts with an hsp70-specific antibody comigrates with Ssc1p that has been imported into mitochondria in vitro.

We have demonstrated previously that *SSC1* is an essential gene (4). At least two other nuclear genes that encode mitochondrial proteins, *MAS1* and *MAS2*, have been shown to be essential (17, 34–36). *MAS1* and *MAS2* encode subunits of the mitochondrial processing protease that catalyzes the cleavage of mitochondrial precursor proteins during import into mitochondria (34). *SSC1*, *MAS1*, and *MAS2* are essential for growth, even on fermentable carbon sources, which is consistent with an essential role for mitochondria in processes other than oxidative phosphorylation, such as fatty acid and nucleotide metabolism.

The predicted amino acid sequence of Ssc1p is clearly related to the sequences of other hsp70 proteins from widely divergent organisms. However, it is interesting that of the hsp70s whose sequences have been determined, Ssc1p is most closely related to DnaK of *E. coli*. For example, in a highly conserved region of 13 amino acids near the amino terminus, Ssc1p has 10 of 13 matches, while Ssa1p and human hsp70 show complete identity (15, 25a). In this same region, DnaK and Ssc1p show 12 of 13 possible matches, with the only mismatch being an isoleucine-to-valine change. The fact that Ssc1p is more closely related to an *E. coli* protein than to other related yeast proteins is consistent with the postulated bacterial origin of mitochondria.

Small amounts of Ssa1p and -2p were present in the mitochondrial preparations used in this study, even though contamination of other cytoplasmic proteins appeared minimal. The presence of Ssa1p and -2p could have been due to the artifactual association of these proteins with mitochondrial preparations, especially since these proteins are very abundant. However, the association of Ssa1p and -2p with mitochondria might reflect an in vivo association of a small portion of Ssa1 or -2p in the cell. It has been shown by genetic analysis that cells lacking *SSA* proteins accumulate precursor forms of at least some mitochondrial proteins (8). It is possible that the mitochondrion-associated Ssa1p and -2p functions both in vitro and in vivo, facilitating protein import.

The identification of Ssc1p as a mitochondrial protein lends support to the idea that hsp70 proteins perform functions in a number of cellular compartments. In cells of many

species, including *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and humans, a major hsp70-related protein is present in the cytoplasm (21, 28, 30). For example, most Ssa1p and -2p is found in the cytoplasm of yeast cells in biochemical fractionation experiments (3). The hsp70-related grp78 protein is found in the endoplasmic reticulum (19); the *KAR2* gene of *S. cerevisiae* encodes an ER-localized member of the yeast family (Rose et al., in press). The finding that Ssc1p is a mitochondrial protein suggests that all major compartments of the cell may contain at least one hsp70-related protein.

The question arises as to whether these proteins perform similar functions in the different cellular compartments. As mentioned above, Ssa1p and -2p are involved in facilitating the transport of at least some proteins into the ER and mitochondria and may have other functions as well. It has been hypothesized that Ssa1p and -2p are involved in altering or maintaining an import-competent conformation of precursor proteins before translocation (8). grp78 is identical to BiP (14), which was first identified as a protein that associated with immunoglobulin heavy chains (13). In normal B cells and plasma cells, a smaller fraction of the intracellular heavy chains is associated with BiP. BiP also binds transiently to a variety of normal secretory and transmembrane proteins and permanently to proteins that are unfolded or misfolded. Because of these data, it has been proposed that grp78 (BiP) plays a role in facilitating the assembly of multimeric protein complexes inside the ER (19). It is tempting to speculate that Ssc1p performs a similar function in the mitochondria, facilitating the assembly of certain protein complexes after translocation across the inner membrane. Strains containing conditional mutations in *SSC1* will be useful in determining the role of Ssc1p in mitochondrial function.

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